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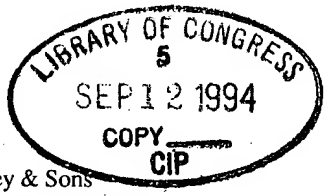

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CHAPTER 4

Use of DNA analysis to determine the diversity of microbial communities

V. Torsvik, J. Goksøyr, F.L. Daae, R. Sørheim, J. Michalsen and K. Salte

Diversity measurements based on the phenotypical characterisation of isolated strains cover only a small fraction of the total information in a microbial community, because only the fraction (0.1-1%) of the bacteria which can grow and form visible colonies on solid media can be analysed. In addition, the phenotypic tests selected account for a small amount of the total information in the DNA. In a study of diurnal fluctuations in river bacteria, Holder-Franklin et al. (1981) compared numerical taxonomy with DNA homology and found that in many cases there was a coincidence between phenetic clusters and genetic relatedness but that some clusters contained more than one species and even more than one genus.

We have determined the DNA diversity in bacterial populations from soil (Torsvik et al., 1990a) and compared genetic and phenetic diversity of populations exposed to stress in the form of increased temperature. We have also studied the complexity of DNA isolated directly from the bacterial community in natural soil (Torsvik et al., 1990b) and compared it with the DNA complexity of a population of isolated bacteria from the same soil. The DNA analysis shows that the genetic diversity of the total microbial community in the soil was about 200 times higher than the diversity of the population of isolated bacteria, and may comprise thousands of species.

ISOLATION OF DNA

Measurements of DNA complexity by reassociation require highly purified DNA, free from eukaryotic DNA, humic material or other impurities. We have developed a method for isolating DNA with high purity directly from bacteria in the soil. The bacteria are first separated from the soil by a fractionated centrifugation procedure (Faegri et al., 1977). The fractionation yield is estimated from the microscopic count, with the number of bacteria in the bacterial fraction as a percentage of the sum of bacteria in the bacterial fraction plus the sediment after the final low-speed centrifugation. Investigators using this counting method have reported yields of 30-35% (Holben et al., 1988; Steffan et al., 1988). In the organic soil being used in this study, the bacterial yield is 60-65% and the bacterial fraction is

substantially free from fungi and other eukaryotic organisms. There are no indications that the fractionation procedure is biased. Our assumption that all the bacterial types are represented in the bacterial fraction is supported by the observation that the yields based on plate count and microscopic count are almost identical (Faegri et al., 1977).

DNA is isolated from the bacterial fraction after removing extracellular DNA and some humic material with hexametaphosphate. The bacteria are lysed with lysozyme and sodium dodecyl sulphate, giving a lysis efficiency of 90-95%. DNA is then extracted from the cells and purified on a hydroxyapatite column (Bio-Gel HT, Bio-Rad Laboratories) (Torsvik, 1980; Torsvik et al., 1990b). The DNA purification causes loss; the highest loss occurs during centifugation, when cell debris and some humic material are removed (30%), and during the hydroxyapatite purification (50%) (Torsvik, 1980). DNA purification does not cause bias; if bias exists, it would lead to a reduction in the DNA complexity.

When starting with 100 g of wet soil (32 g dry weight) with 4.8×10^{11} bacteria, the DNA yield is 3.5-5.0 µg/g wet soil. Assuming an average DNA amount per bacterial cell of 5×10^{-15} g (Bak et al., 1970), the theoretical yield is 24 µg/g wet soil. Thus, 15-20% of bacterial DNA is recovered from the soil.

DETERMINATION OF GENETIC DIVERSITY

Genetic diversity is a measure of the number and frequency distribution of genetically different bacteria in a bacterial population or community. It can be determined from the complexity of DNA extracted from the mixture of bacteria. DNA complexity is defined as the total length of different DNA sequences measured in number of base pairs (bp) in a defined amount of DNA. This is an ideal quantity and is equal to the genome size for haploid cells with single-copy DNA. The kinetic DNA complexity is a measurable quantity calculated from the reassociation rate of sheared and melted (single-stranded) DNA in solution (Britten and Kohne, 1968). The reassociation of homologous single-stranded DNA follows second-order reaction kinetics where the reaction rate is proportional to the square of the concentration of homologous DNA strands. The fraction of reassociated DNA is expressed as a function of C_0t (C_0 is the molar concentration of nucleotides in single-stranded DNA at the beginning of the reassociation, and t is the time in seconds). The reaction rate constant depends upon the relative concentration of complementary DNA sequences and is proportional to the reciprocal of C_0t for half reassociation ($1/C_0t_{1/2}$). Under defined conditions (for example, cation concentration, temperature, DNA fragment length and concentration) $C_0t_{1/2}$ is thus proportional to the complexity of the DNA.

We normally measure the reassociation of sheared (French Press at 20 000 psi) and melted DNA spectrophotometrically in 4-6 x standard saline citrate (SSC) with 30% dimethylsulphoxide (DMSO) added (Torsvik et al., 1990a), using the *Escherichia coli* genome as a reference.

The reassociation curves for DNA from mixtures of bacteria have flatter slopes than that corresponding to an ideal second-order reaction. This may arise from an uneven distribution of the different DNA fractions in the mixture, which gives several second-order reactions with different rate constants. In this case, $C_0t_{1/2}$ has no precise kinetic meaning, but it still can be used as a parameter for expressing the complexity of DNA from mixed bacterial populations and communities.

The genetic diversity of a bacterial community can be expressed by using $C_0t_{1/2}$ under defined conditions as a diversity index. The concept of genetic diversity as we use it corresponds to that used in information theory, where diversity is a measure of the total amount of information in a system (that is, a community) and the distribution of this information (the amount of information found in a few individuals, and the amount found in a large number of individuals). The diversity index $C_0t_{1/2}$ is analogous to the Shannon Weaver index, which is based on numeric taxonomy clusters or species diversities (Atlas, 1984).

APPLICATIONS OF THE DNA REASSOCIATION METHOD

We have used the DNA reassociation method to study the effect of external stress factors on the diversity of bacterial communities, and to compare the diversity of cultured bacteria with diversity of the total bacterial population in soil.

Temperature stress and diversity

In a model study, the diversity indices based on genetic and phenetic measures were compared, and the usefulness of these indices in demonstrating the effect of an external stress factor on the bacterial community was tested. Therefore, only populations of culturable bacteria from the soil were included in the study.

Our model system was a beech forest soil in western Norway (Sørheim et al., 1989). The microscopic count (acridine orange stained) was 1.5×10^{10} and the plate count (Thornton's medium with 10% soil extract; Thornton, 1922) was 4.2×10^7/g dry soil. The soil was stored at 4°C and 30°C for 3 months. By the end of this time the total counts had decreased to 2.5×10^9/g dry soil for the soil stored at 4°C, and to 3.5×10^9/g dry soil for the soil stored at 30°C. The plate counts of the two soils were 2.2×10^7/g dry soil and 5.5×10^7/g dry soil, respectively.

For phenotypic and genotypic testing, 80 strains were selected randomly from the isolates from each soil. The isolates were derived from colonies selected at random from plates used for plate counts. For phenotypic testing, a set of 26 physiological (API 20B and API OF systems; API System S.A., France) and morphological tests were implemented. The proximity between strains was calculated (simple matching coefficient) and they were clustered (complete link, furthest distance method; Sørheim et al., 1989). The dendrograms of the 4°C and 30°C strains (*see* Figures 4.1 and 4.2 *overleaf*) show that the structure of the two populations was entirely different. When a similarity level of 80% was used to define the biotypes, the two populations contained 35 biotypes (*see* Table 4.1). The 4°C population contained 33 biotypes with few strains in each. The 30°C population contained 12 biotypes, two of which were dominant, and the most abundant contained 61% of all the strains in the population.

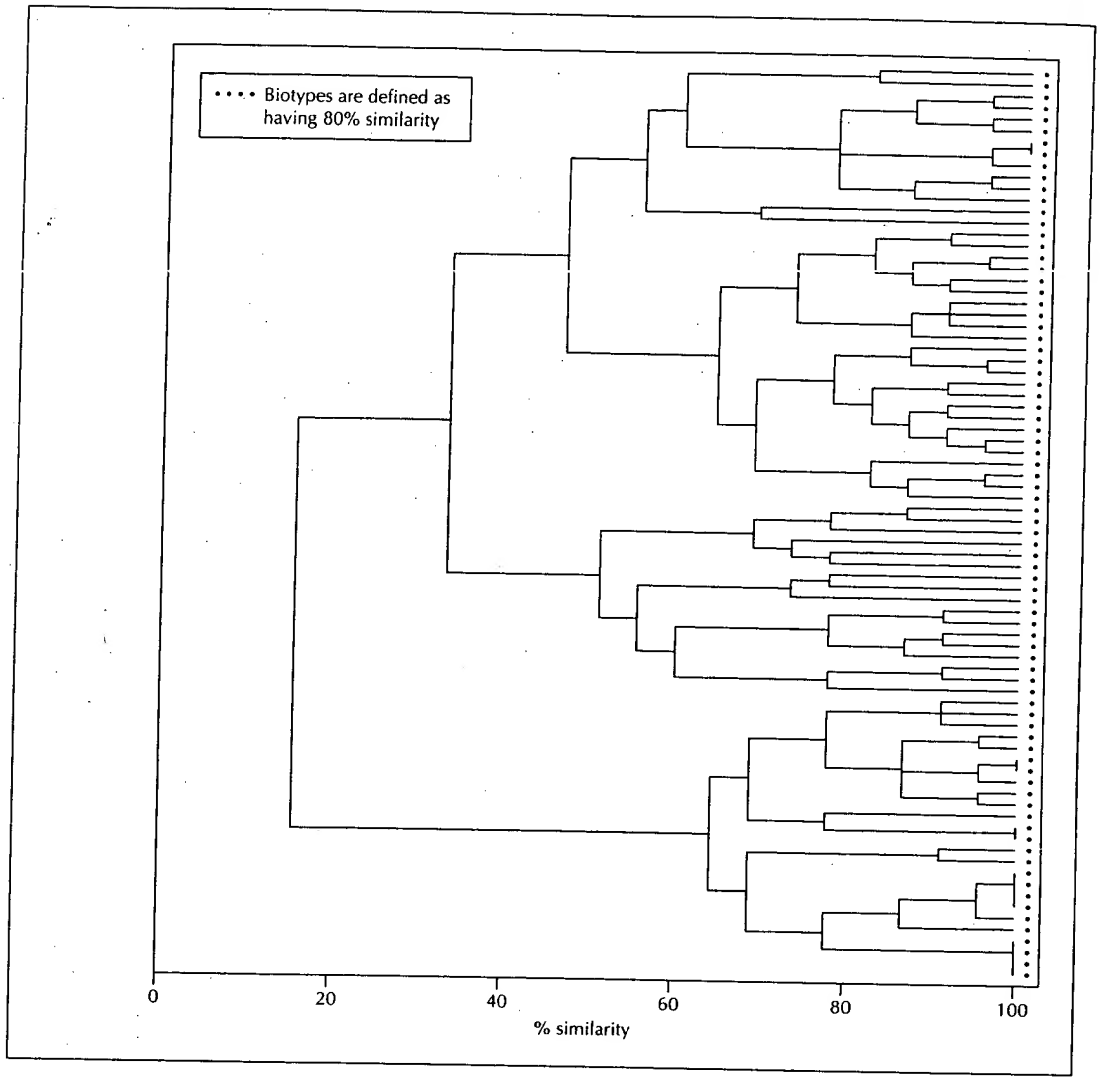
The phenetic diversity was also expressed as cumulative difference, as described by Torsvik et al. (1990a). This was done by correlating the number of strains in populations of increasing size with the

Table 4.1 **Numbers of biotypes, cumulative difference, Shannon index (H' logarithmic base), equitability ($J = H'/H'_{max}$) and population diversity index ($C_0t_{1/2}$) for the 4°C and 30°C populations separately and combined**

Population	4°C	30°C	4°C + 30°C
Number of strains	80	80	160
Biotypes	33	12	35
Cumulative difference	121	28	133
H'	4.79	2.11	4.07
J	0.95	0.59	0.79
$C_0t_{1/2}$[a]	34.7	5.8	22.4

Note: a In 4 x standard saline citrate (SSC), 30% dimethylsulphoxide (DMSO)

Figures 4.1 **Cluster analysis (complete link, furthest neighbour) of the bacterial population isolated from soil stored at 4°C**



number of different phenotypes or differences in test results. The strains were placed in a randomly chosen sequence and for each new strain selected, the phenotypic test results were compared with those for all previously selected strains. The smallest differences thus obtained represented new phenetic information in the new strain. The sum of the differences was plotted against the number of strains added. An error margin of 2 was included to account for misreading of test results. The cumulative differences for the 4°C and 30°C strains are shown in Figure 4.3 (*overleaf*).

The increase in genetic diversity with an increasing number of strains was determined by DNA reassociation. DNA was isolated using the method described by Marmur et al. (1963), and to reduce the work involved the isolation was performed from groups of 10 strains lumped together. The strain

population isolated

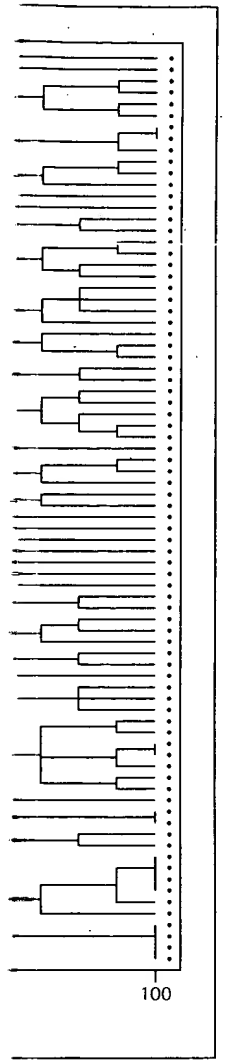


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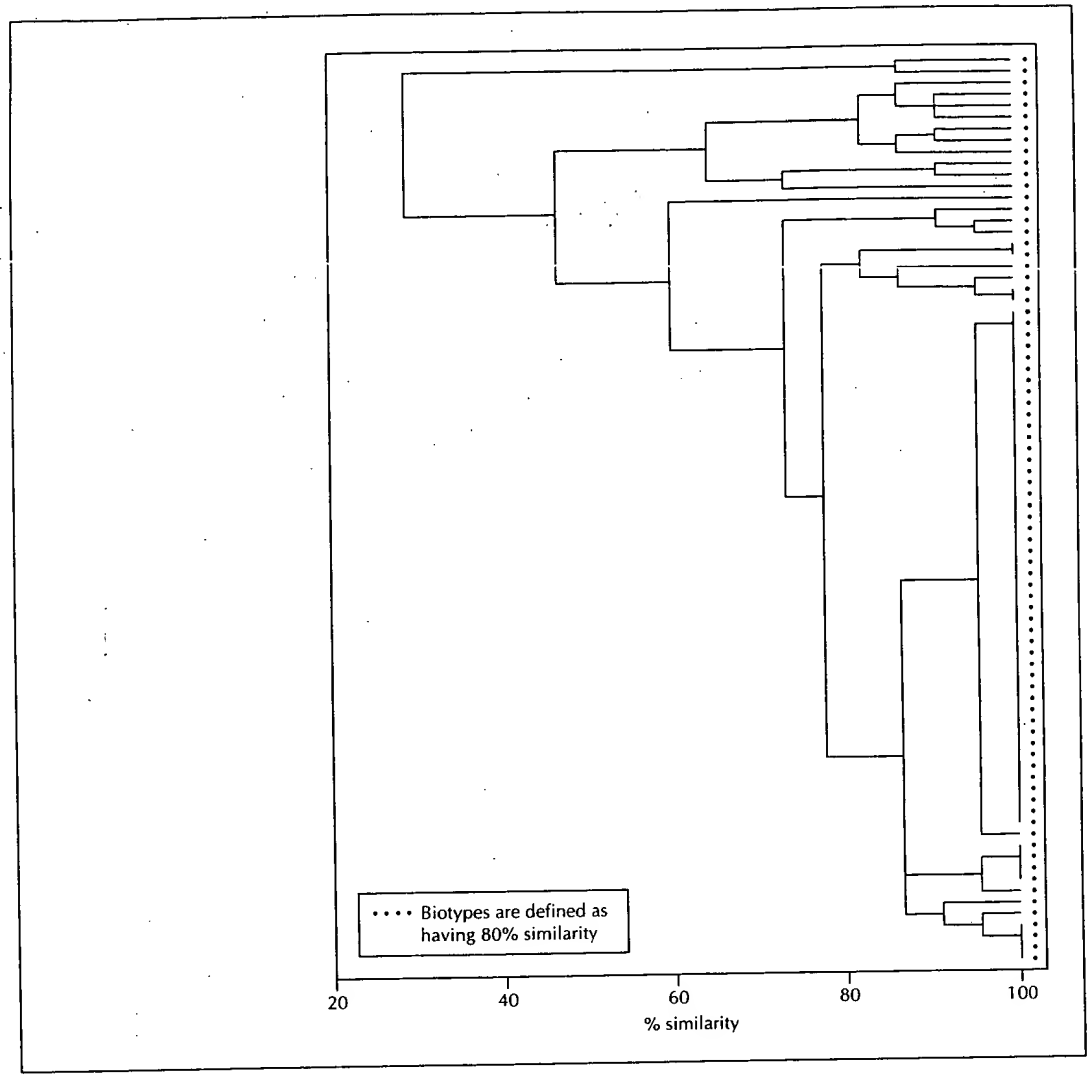
Figure 4.2 **Cluster analysis (complete link, furthest neighbour) of the bacterial population isolated from soil stored at 30°C**



sequence was the same as that used for determining cumulative difference. DNA from group 1 was reassociated first, and the reaction rate expressed as $C_0t_{1/2}$ was determined. Equal amounts of DNA from group 1 and group 2 were then mixed and reassociated, and $C_0t_{1/2}$ for this mixture was determined. This process was repeated until all the groups had been included in the reassociation mixture. The $C_0t_{1/2}$ for each reassociation mixture was plotted against the number of strains added (*see* Figure 4.4 *overleaf*). The amount of genetic information in the 4°C and 30°C populations was very different. Figure 4.5 (*overleaf*) shows the reassociation curve for the mixture of DNA from the 4°C and 30°C populations combined (160 strains), compared with the curves for DNA from these two populations individually.

Figure 4.3 **Cumulative differences for bacterial populations isolated from soil stored at 4°C and 30°C**

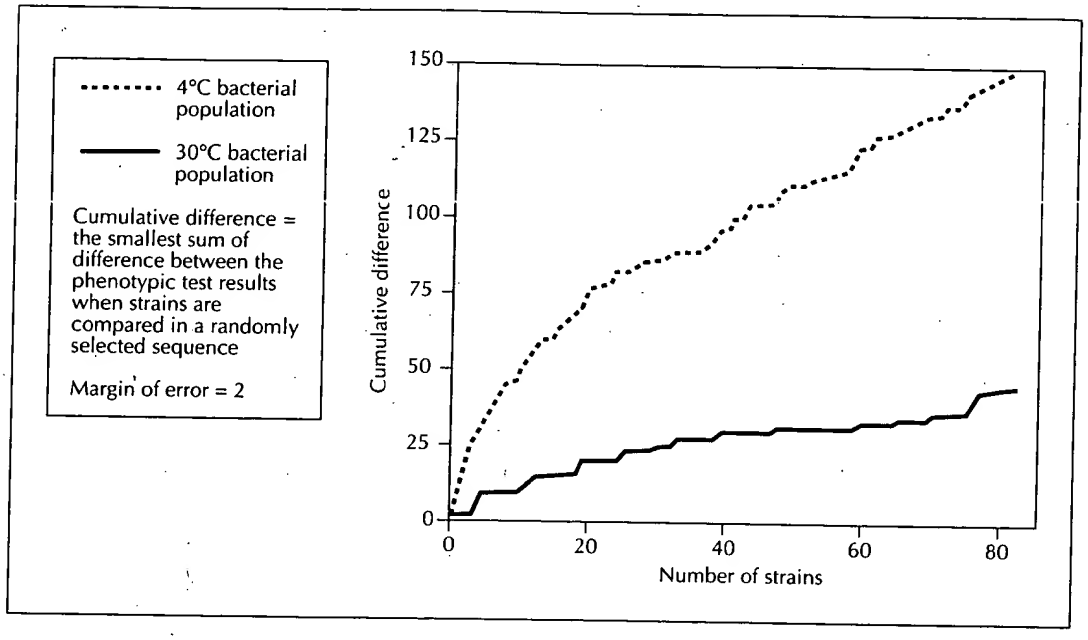
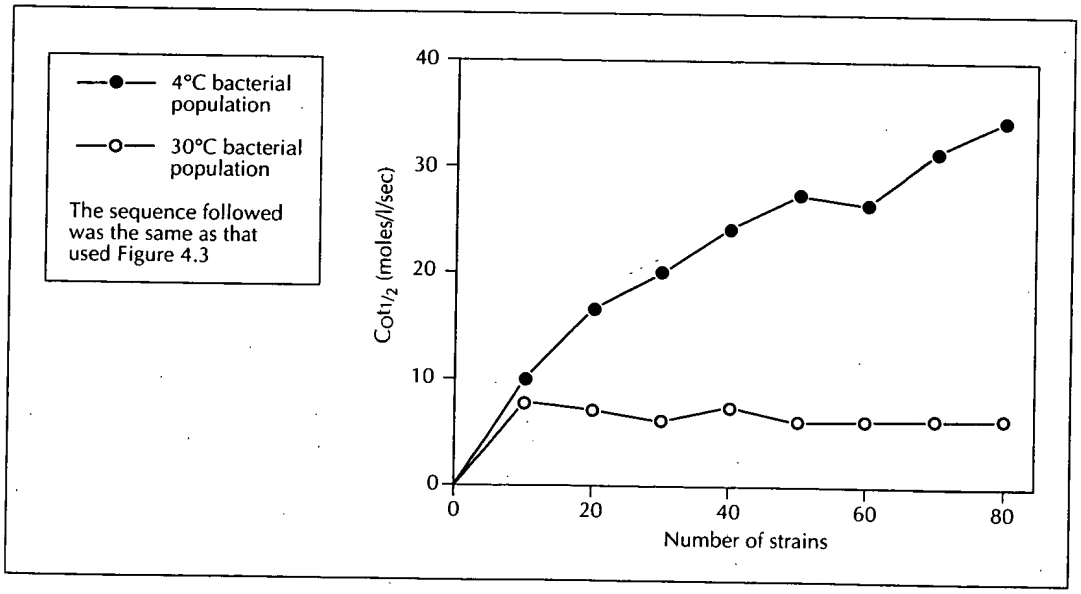
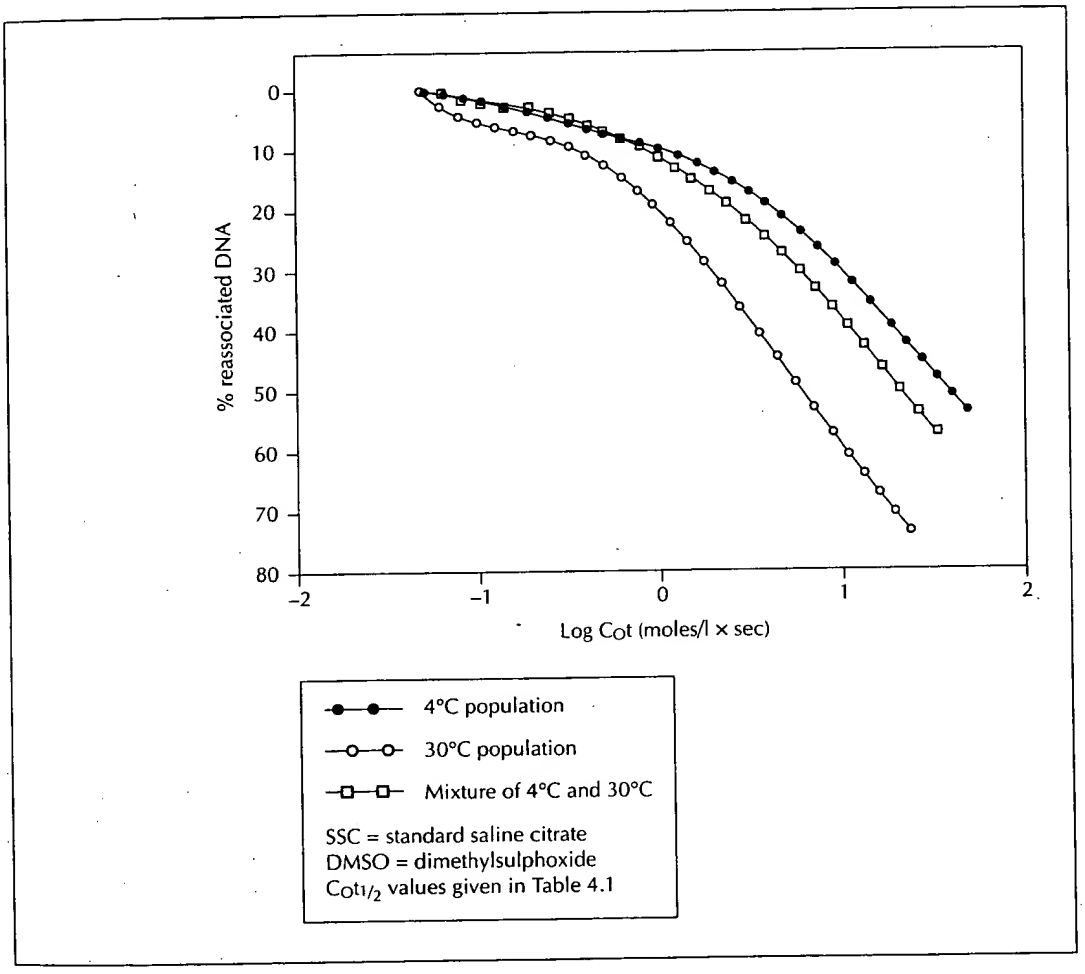


Figure 4.4 **The diversity index ($C_O t_{1/2}$) values with increasing numbers of bacterial strains isolated from soil stored at 4°C and 30°C**



There seems to be a general relationship between DNA diversity and phenotypic diversity in this model system. Under the experimental conditions, the average $C_0t_{1/2}$ for soil bacteria was about 1.4 moles/l x sec, some 60% higher than $C_0t_{1/2}$ for *E. coli* DNA (Torsvik et al., 1990a). In the 4°C population containing 33 biotypes, $C_0t_{1/2}$ for all the 80 strains was 35 moles/l x sec. For the 30°C population containing 12 biotypes, $C_0t_{1/2}$ was 6 moles/l x sec. The curves for cumulative difference and genetic diversity had the same overall appearance, and indicated that the reduction in phenetic and genetic diversity was due to the higher temperature. However, there is a difference in the two diversity measurements, most evident in the curves for the 30°C population. The cumulative difference increased with increasing numbers of strains, but the $C_0t_{1/2}$ levelled off at 10 strains. The reason is that the cumulative difference measures only the amount of information in the population, while the genetic diversity also takes into account how this information is distributed within the population.

Figure 4.5 **Reassociation curves at 51°C in 4 x SSC, 30% DMSO for all bacterial strains from soil stored at 4°C and 30°C, separately and combined**



When a mixture of DNA from the two populations was reassociated, the $C_0t_{1/2}$ fell between the $C_0t_{1/2}$ for the two populations reassociated separately. The mixture of the two populations has more biotypes and a higher cumulative difference (*see* Table 4.1), but this was counterbalanced by the increasing number of identical isolates from the 30°C population, increasing the concentrations of homologous DNA in the mixture relative to that of the 4°C population. The $C_0t_{1/2}$ values change in the same manner as the Shannon Weaver index or Equitability (*see* Table 4.1), which also measures both the amount and the distribution of information in the populations.

Diversity of the total bacterial community

The genetic diversity of the total bacterial community in the soil was compared with the genetic diversity of a population of isolated bacteria. The experiments have been described by Torsvik et al. (1990a, b). About 200 strains were randomly chosen from plates of a standard plating medium, and isolated. This population contained 41 biotypes at 80% phenotypic similarity. Figure 4.6 shows the reassociation of DNA from a mixture of the isolated strains, compared with the reassociation of DNA derived directly from the bacterial fraction of the same soil.

The $C_0t_{1/2}$ of DNA from the mixture of the isolated bacterial strains was 28, corresponding to a DNA complexity of 1.4×10^8 bp. The DNA complexity seemed to have reached its maximum value at approximately 90 strains (Torsvik et al., 1990a). The $C_0t_{1/2}$ of DNA isolated from the total bacterial fraction of the same soil was 4700, corresponding to 2.7×10^{10} bp (*see* Table 4.2). Thus, the genetic diversity of the total bacterial population was about 200 times higher than that of the isolated bacteria.

According to a proposal put forward by the committee on the reconciliation of approaches to bacterial systematics (Wayne et al., 1987), strains with at least 70% DNA homology can be defined as belonging to the same species. The maximum value for the number of species would therefore be 3.3 times the number of genomes with no homology. The isolated bacteria therefore consist of a maximum of 66 species with 70% homology, while the maximum number of species in the total bacterial community is approximately 13 000. This indicates that the bacterial types that can be isolated using the standard plating technique are only a small fraction of the soil bacterial population.

Our findings are supported by analysis of 16S ribosomal RNA (rRNA) from environmental samples (Giovannoni et al., 1990; Ward et al., 1990; Fuhrman et al., 1992). It has been found that the 16S rRNA isolated directly from environmental samples does not correspond to that from isolated organisms, and the conclusion drawn was that there may be high genetic variability within natural microbial communities.

It may be difficult to understand that up to 10 000 bacterial species can be harboured in 100 g of soil. The total number of bacteria in 100 g wet soil was about 5×10^{11}. If we assume an even species distribution, each species would consist of about 5×10^7 individuals. Using the immunofluorescence technique, it has been demonstrated that the population size of three specific bacteria in an organic soil ranged from 10^6 to 10^7 cells/g of soil (Sjåstad, 1979). The average plate counts for the three bacteria were about 0.5% of the immunofluorescence counts. Even if 99.5% of the cells were in a non-culturable state, this is an ample population size for species sustainability. Rare species present in frequencies far below the average still have a fairly high population size.

How can so many species have evolved? The high complexity of DNA from soil bacteria may reflect a vast amount of genotypically separate clones in soil, most of them probably unknown. Environments such as soil may contain a large number of different ecological niches which can sustain a great variability of the bacterial flora utilising a combination of substrates under different physico-chemical conditions.

Figure 4.6 **Reassociation of DNA from the soil bacterial fraction, 206 isolated bacteria from the same soil and *Escherichia coli* in 4 x SSC and 30% DMSO**

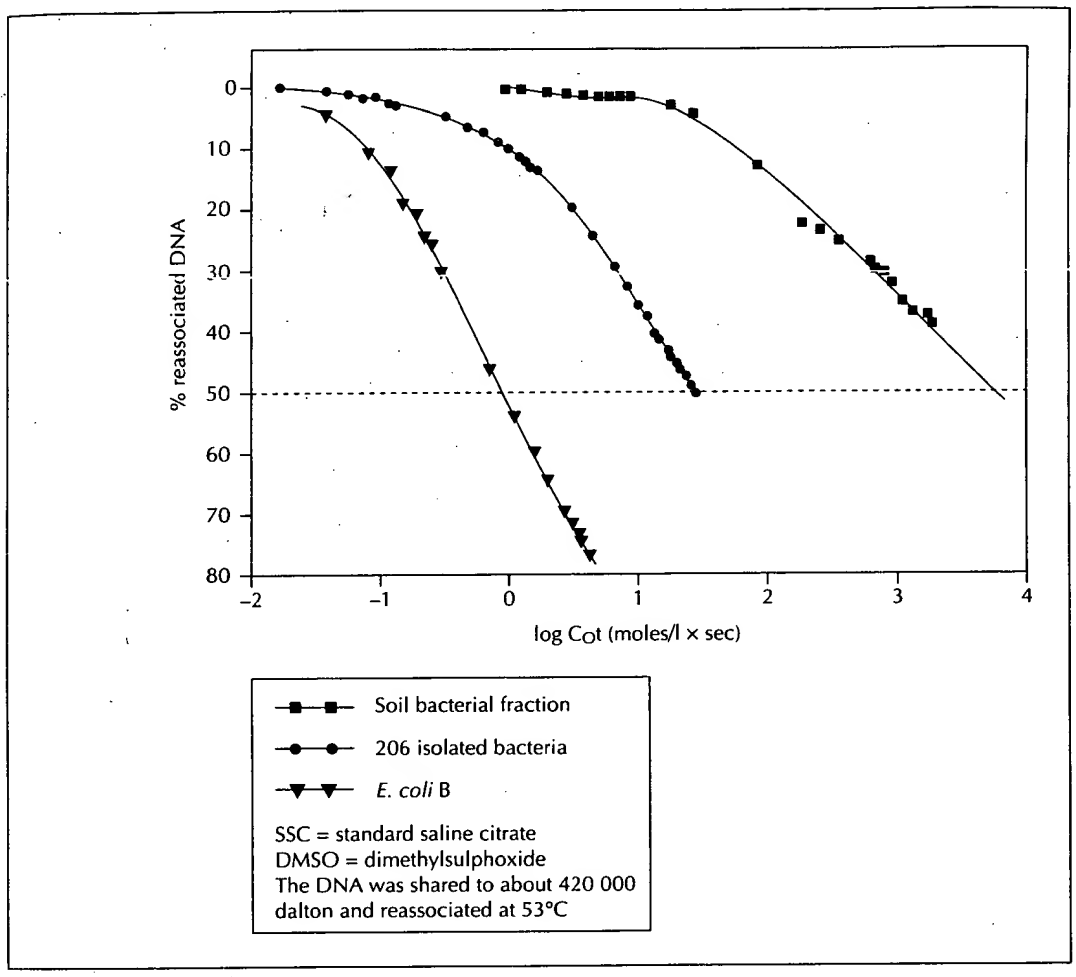


Table 4.2 **Mole % G + C, reassociation kinetics ($C_0t_{1/2}$; moles/l x sec at half reassociation), complexity in base pairs (bp) and numbers of standard soil bacterial genomes (6.8×10^6 bp)**

DNA source	Mole % G + C	$C_0t_{1/2}$	Complexity (bp)	Standard genomes
Escherichia coli	50.7	0.85[a]	4.1×10^6	0.6
E. coli	50.7	0.72[b]	4.1×10^6	0.6
206 isolates	59-65	28	1.4×10^8	20.6
Bacterial fraction	58.5	4700[b]	2.7×10^{10}	4000

Note: a In 4 x standard saline citrate (SSC), 30% dimethylsulphoxide (DMSO)
b In 6 x standard saline citrate (SSC), 30% dimethylsulphoxide (DMSO)

CONCLUSION

For populations of isolated bacteria, there is good general agreement between phenetic diversity indices based on a limited number of phenotypic characters and genetic diversity based on DNA complexity, measured as reassociation rates and expressed as $C_0t_{1/2}$. The genetic diversity expressed as $C_0t_{1/2}$ is a good parameter for revealing the differences between a highly diverse population and one in which diversity has collapsed. The genetic diversity can be determined from the complexity of DNA isolated directly from the bacteria in soil, and is currently the only method for assessing the diversity of the total bacterial community. The diversity of this community in soil is very much higher than expected from work with cultured bacteria. Most of the bacteria in these environments are unknown.

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